Involvement of Transforming Growth Factor-β in the Formation of Fibrotic Lesions in Carcinoid Heart Disease

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Carcinoid heart disease is a complication of a neuroendocrine carcinoid tumor. Morphologically, it is characterized by the formation of fibrotic plaques with deposition of extracellular matrix in the subendocardium, frequently causing heart valve dysfunction and cardiac failure. Because members of the transforming growth factor-β (TGF-β) family are known to stimulate fibroblasts in their production of extracellular matrix, we investigated the expression of the three isoforms of TGF-β and the binding protein for latent TGF-β1 (LTBP) in carcinoid plaques of the right side of the heart, as well as from control tissue, using immunohistochemistry. Tissue specimens were obtained intraoperatively from nine consecutive patients undergoing valve replacement surgery. TGF-β1 and TGF-β3 were detected in the fibroblasts of all plaques analyzed, whereas TGF-β2 was only rarely expressed. The localization of LTBP was partly concordant with that of TGF-β1, but the positive staining for LTBP was extracellular. Sections from unaffected heart tissue contained few fibroblasts in the subendocardium, showing only weak or no immunostaining for TGF-β1, -β2, and -β3 and no staining for LTBP. These results suggest that TGF-β may play a role in the proliferation of fibroblasts and their matrix production in carcinoid heart lesions. (Am J Pathol 1993, 142:71–78)

Carcinoid heart disease is a unique complication of a malignant, neuroendocrine tumor, which is usually localized within the gastrointestinal tract and characterized by endocrinologically active, enterochromaffin cells. Carcinoid heart disease represents a serious clinical condition, and one-third of all deaths among patients with carcinoid syndrome are related to right ventricular failure secondary to morphological changes in the heart.[1] Echocardiographic signs of carcinoid heart disease is found in two thirds of these patients.[2,3] The carcinoid heart lesions are located on the mural and valvular endocardium predominantly in the right side of the heart. They consist of fibroblasts or myofibroblasts and a matrix-rich fibrous stroma devoid of elastic fibers and are covered by endothelium.[4,5] The prevalence of carcinoid heart disease is increasing due to prolonged life expectancy of the patients, secondary to improved treatment protocols of the carcinoid tumor.

Although several suggestions have been proposed, the etiology of the lesions is still unknown. The lack of an experimental animal model complicates this task. However, a positive correlation with the levels of circulating vasoactive substances such as serotonin and tachykinins has been demonstrated.[2] This might indicate that substances produced by the carcinoid tumor participate directly or indirectly in the development of these fibrotic lesions.

A possible candidate involved in this severe fibroproliferative process could be the transforming growth factor-β (TGF-β), which—among a variety of different functions (reviewed in ref 6)—affects growth and differentiation of cells and stimulates fibroblasts to produce extracellular matrix components.[7] In fact, increased levels of TGF-β have been found in myofibroblasts and fibroblasts in carbon tetrachloride-induced liver fibrosis in rats.[8] TGF-β is a family of

structurally related isoforms, TGF-β1, -β2, and -β3, that are synthesized and secreted as large, latent complexes. In these complexes TGF-β is noncovalently associated with the N-terminal remnants of their precursors (latency associated peptides [LAPs]); TGF-β1 complexes from certain sources, eg, platelets, have been shown to contain also a third component denoted latent TGF-β1 binding protein (LTBP),[9] the functional role of which remains to be elucidated (for reviews, see refs 6 and 10). To exert their biological effects via binding to specific cell-surface receptors, TGF-βs have to be activated; the exact mechanisms for activation are not known, but may involve enzymatic degradation of the LAP molecules of the precursors. To explore the possibility that TGF-βs are involved in the development of the carcinoid heart disease, we investigated whether the precursor molecules of TGF-β1, TGF-β2, and TGF-β3 as well as LTBP are present in the cardiac lesions of patients suffering from carcinoid syndrome.

Materials and Methods

Patient Material

Cardiac tissue was obtained from nine consecutive patients with classical carcinoid syndrome due to metastasing mid-gut carcinoid tumors, who were referred to cardiac surgery because of severe carcinoid heart disease. The patients (five female, four male) had a median age of 60 years (range, 43 to 72). At the time of cardiac surgery an average of 5 years (range, 1 to 13) had elapsed since they experienced their first carcinoid symptom. The carcinoid diagnosis was confirmed histologically and biochemically 9 months to 10 years preoperatively. Treatment with α-interferon and octreotide had resulted in clinical control of the disease in all but one of the patients (patient 1). Valve replacement surgery of the tricuspid and pulmonary valves was performed in all patients because of progressive right ventricular failure. Tissue specimens of the fibrotic tricuspid valves and papillary muscles, pulmonary valves, and right atrial wall were fixed in 4% paraformaldehyde, and embedded in paraffin, and 6-μm sections were placed on glass slides.

Normal Material

Operative and autopsy specimens from various parts of the right sides of normal hearts served as control tissues. They were fixed and treated in the same way as the patient material.

Cell Culture

The human glioblastoma cell line U-1240 MG[11] was cultured in chamber slides (Lab-Tek, Nunc Inc, Naperville, IL) using Dulbecco's modified Eagle's medium containing 10% fetal calf serum. When the cells reached a subconfluent state, the slides were fixed in ice-cold acetone for 10 min, air-dried, and stored at −70 C. The U-1240 MG cells served as a positive control for all the antibodies tested in this study.[11a]

Antisera

Polyclonal rabbit antisera were raised as previously described.[12] Ab 39 was raised against LTBP purified from human platelets.[9] The TGF-β antisera were raised against synthetic peptides corresponding to variable regions of the three TGF-β precursors: anti-β1-LAP against the amino acids 262–277 of β1-LAP, anti-β2-LAP against the amino acids 285–300 of β2-LAP, and anti-β3-LAP against the amino acids 282–298 of β3-LAP.[11a]

The three peptide antisera were loaded on affinity columns containing the corresponding synthetic peptides coupled to CH-Sepharose 4B (Pharmacia LKB, Uppsala, Sweden), and the bound antibodies were eluted at pH 3, neutralized, and dialyzed against phosphate-buffered saline with 10% glycerol.

Immunohistochemistry

Paraffin-embedded sections were dewaxed in xylene and hydrated through standard graded ethanol solutions. Endogenous peroxidase activity was quenched by 0.3% hydrogen peroxide in methanol. An avidin-biotin-blocking kit (Vector Laboratories, Burlingame, CA) was applied to prevent a nonspecific reaction between endogenous biotin and the detection system. The ABC technique[13] was performed using Vectastain ABC kit (Vector Laboratories) for detection of immunoreaction with the rabbit antibodies. The sections were weakly counterstained with methyl green.

The stained tissue sections were independently viewed by three of the authors. A semiquantitative scheme, specifying the intensity of the immunostaining as well as the number of stained cells within the carcinoid plaques, was used to evaluate all the tissue sections. (For details, see the legend to Table 1.)

Results

Tissue specimens from various locations of the right side of the heart, taken from nine patients undergo-

Table 1 *Immunostaining of Fibroblasts in Carcinoid Plaques and in Normal Subendocardium for the Various Precursors of TGF-β and LTBP*

Patient	Tissue	Anti–β1-LAP	Anti–β2-LAP	Anti–β3-LAP	Anti–LTBP
1	Tricuspid valve	(+)	++	++	–
	Right atrial wall	–	–	+	–
	Pulmonary valve	(+)	+	++	–
2	Tricuspid valve	+	(+)	++(+)	–
	Right atrial wall	+	–	+	–
3	Tricuspid valve	–	(+)	+	–
	Right atrial wall	(+)	0	+	–
4	Papillary muscle	+(+)	+	++	++
	Tricuspid valve	+	(+)	++	+
	Right atrial wall	(+)	(+)	0	–
5	Papillary muscle	++	(+)	++	–
	Tricuspid valve	+++	–	++	–
6	Papillary muscle	+	(+)	++	+
	Tricuspid valve	++	–	+++	–
7	Papillary muscle	–	–	+	–
	Right atrial wall	+	–	++	–
8	Papillary muscle	+	(+)	+(+)	–
	Tricuspid valve	+	(+)	++	–
9	Tricuspid valve	+	–	++	(+)
	Right atrial wall	++	–	+(+)	+
Control	Papillary muscle	(+)	–	–	–
	Tricuspid valve	–	–	(+)	–
	Right atrial wall	(+)	(+)	(+)	–
	Pulmonary valve	–	(+)	(+)	–

* Immunostaining is located intracellularly in the case of anti–β1-LAP, anti–β2-LAP, and anti–β3-LAP, extracellularly in the case of anti-LTBP. Semiquantitative evaluation of immunostaining; –, no immunostaining; +, few positive cells; ++, less than 50% of the cells positive; +++, more than 50%, but not all cells positive; ++++, all cells positive; ( ), weak staining; 0, plaque not present.

ing valve replacement surgery for severe carcinoid heart disease, were analyzed. The typical carcinoid plaques were found in all the different locations examined (Table 1). Corresponding specimens of unaffected hearts served as normal controls.

Immunostaining of the cultured human glioblastoma cell line U-1240 MG was used as a positive control for the different primary antibodies applied to the sections. The concentrations giving the optimal staining were used on the carcinoid heart tissue. Specificity of the immunostaining was demonstrated by blocking experiments: The positive staining of the affinity-purified antisera for the various TGF-β precursors was greatly reduced when they were preincubated with an excess of the peptides against which they were raised, respectively. The polyclonal antiserum Ab 39 was neutralized by preincubation with a 10-fold molar excess of the purified LTBP in the same way. Staining with the preimmune serum was negative. The possibility of unspecific staining caused by the detection system can be excluded because replacement of the primary antibody by 10% normal goat serum resulted in no staining (data not shown).

Specific immunostaining for the various TGF-β precursors was found in the cytoplasm of the fibroblasts within the carcinoid plaques. Whereas the immunostaining for TGF-β1 was moderate and only detectable inside a limited number of cells within

each plaque (Figure 1A), the staining for TGF-β3 was in general rather strong and present in an average of nearly 50% of the cells (Figure 1E). The number of TGF-β1 positive cells, however, showed—compared with that for TGF-β3—a larger variation between different patients and ranged from a weak or negative staining in patients 1 and 3 to a strong and widespread staining pattern in the tissue from patient 5 (Table 1). The staining for TGF-β2 was generally weak or undetectable and never involved more than one-third of the cells within the plaques (Table 1, Figure 1C). Normal heart specimens contained some fibroblasts within the subendocardial connective tissue. Their cytoplasm showed either no or only a weak immunoreactivity for the various TGF-β isoforms (Figure 2A). Among the specimens containing weak TGF-β immunoreactivity, never more than one-fourth of the unstimulated fibroblasts were positive.

Strikingly, the superficial part of the plaques, the area adjacent to the endocardium, showed in most of the specimens a stronger staining than did the area underneath, next to the cardiac muscle or the stroma of the valve (see Figure 1A, 1C). Besides the staining within the fibroblasts of the carcinoid plaque, distinct immunostainings for all three TGF-β precursors were seen in the endocardium (Figure 1A, 1C, 1E), in endothelial cells (Figure 1A, 1C), and in the cardiac muscle (Figure 1A, 1C, 1E). The presence of TGF-β
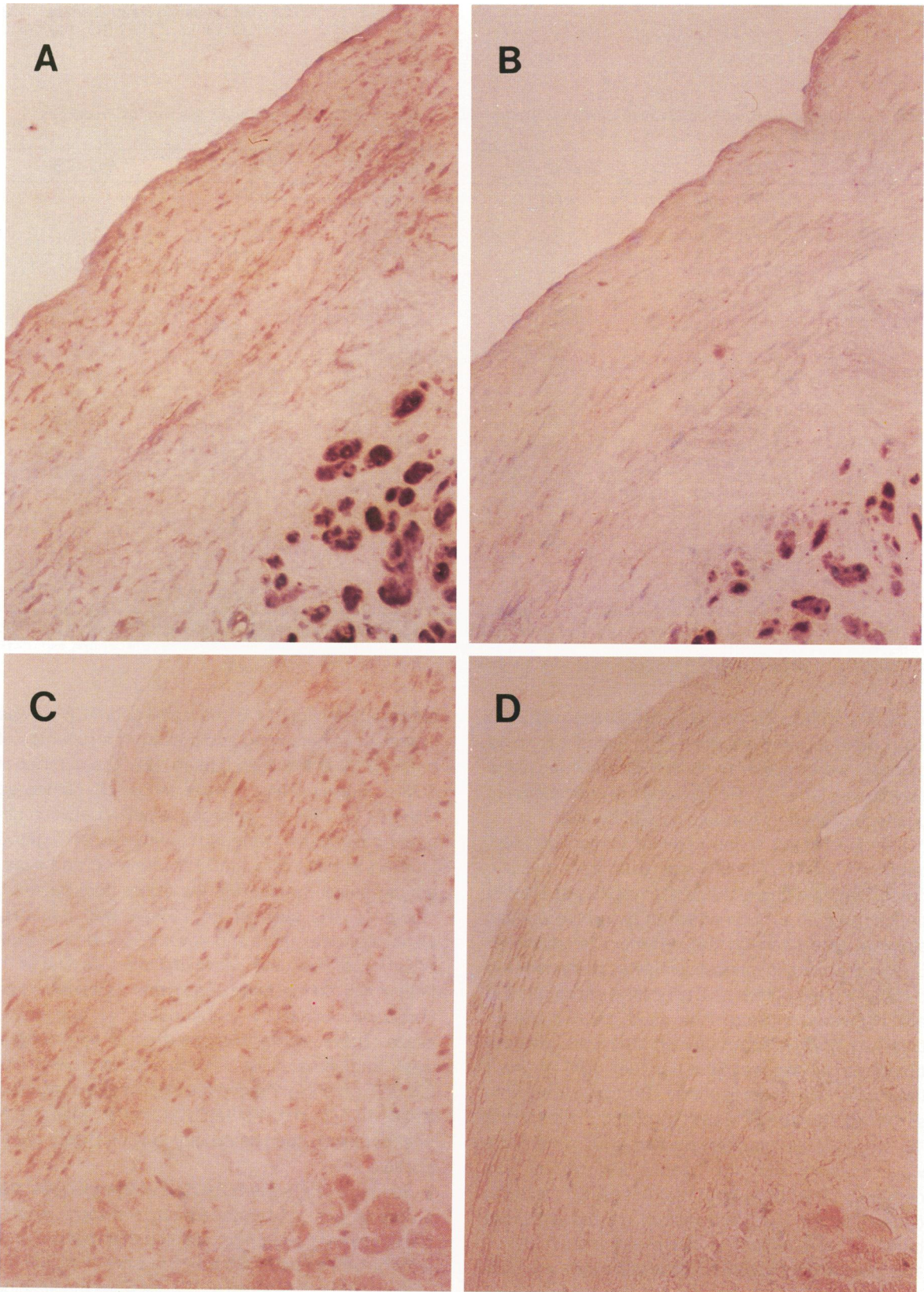
Figure 1. *Representative case of a carcinoid plaque of the right atrium (papillary muscle from patient 4) containing the latent forms of TGF-β1, TGF-β2, TGF-β3, and LTBP. The immunoreactivity was demonstrated by the ABC-immunoperoxidase staining method. Whereas β1-LAP (A), β2-LAP (C), and β3-LAP (E) are located inside the immature fibroblasts of the plaque, LTBP (G) is located extracellularly. Specificity of the immunostaining for β1-LAP (B), β2-LAP (D), β3-LAP (F), and LTBP (H) is shown by blocking of the immunoreactivity by preincubation of the diluted antiserum with a 10-fold molar excess of peptide/protein used for immunization of the rabbits. Blocking resulted in a significant decrease of the specific immunostaining. Immunostainings in cardiac muscles are stronger than those in the plaque fibroblasts, and considerable immunoreactivity still remained after the blocking. All the sections were counterstained by methyl green. Original magnification is × 250.*
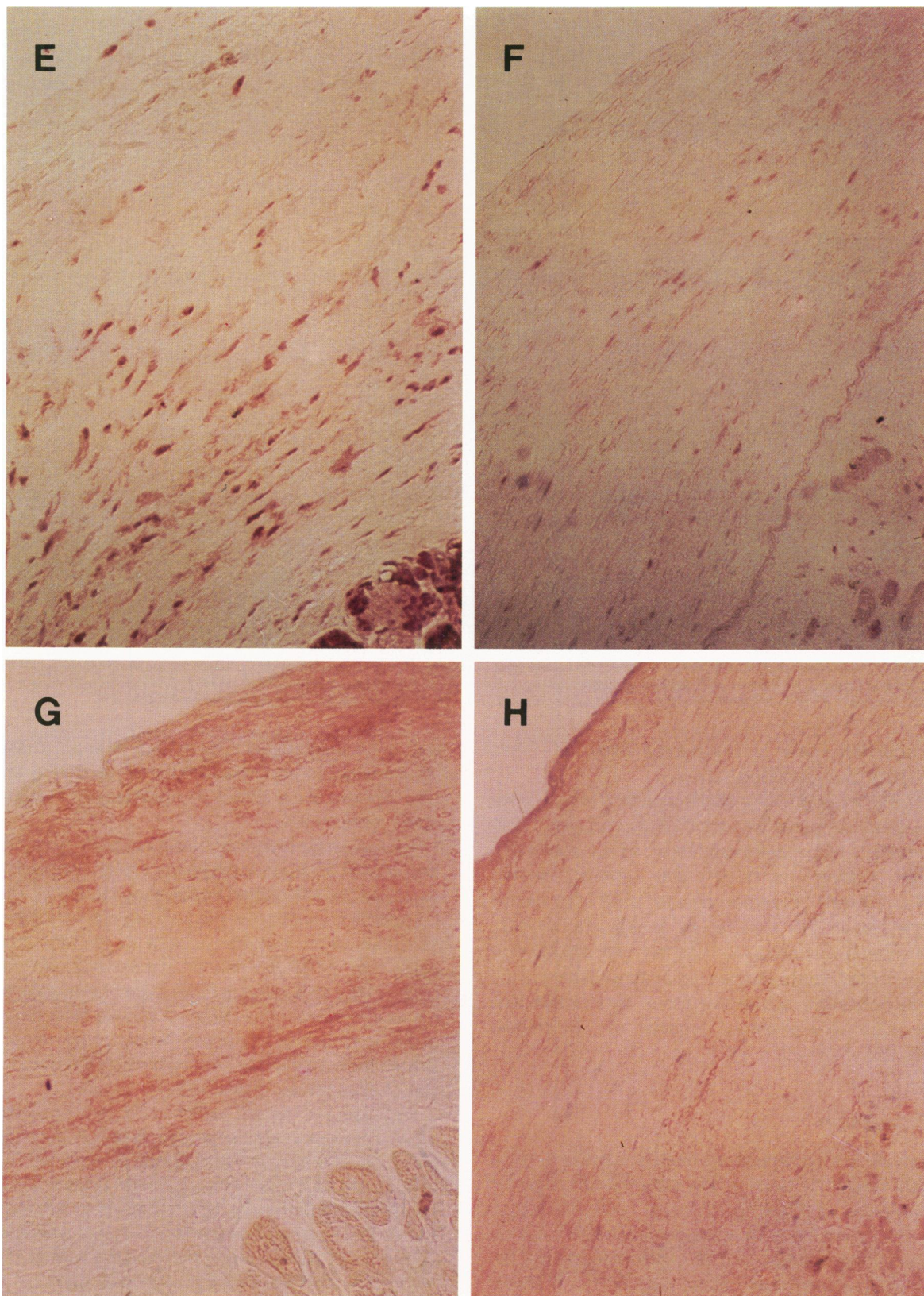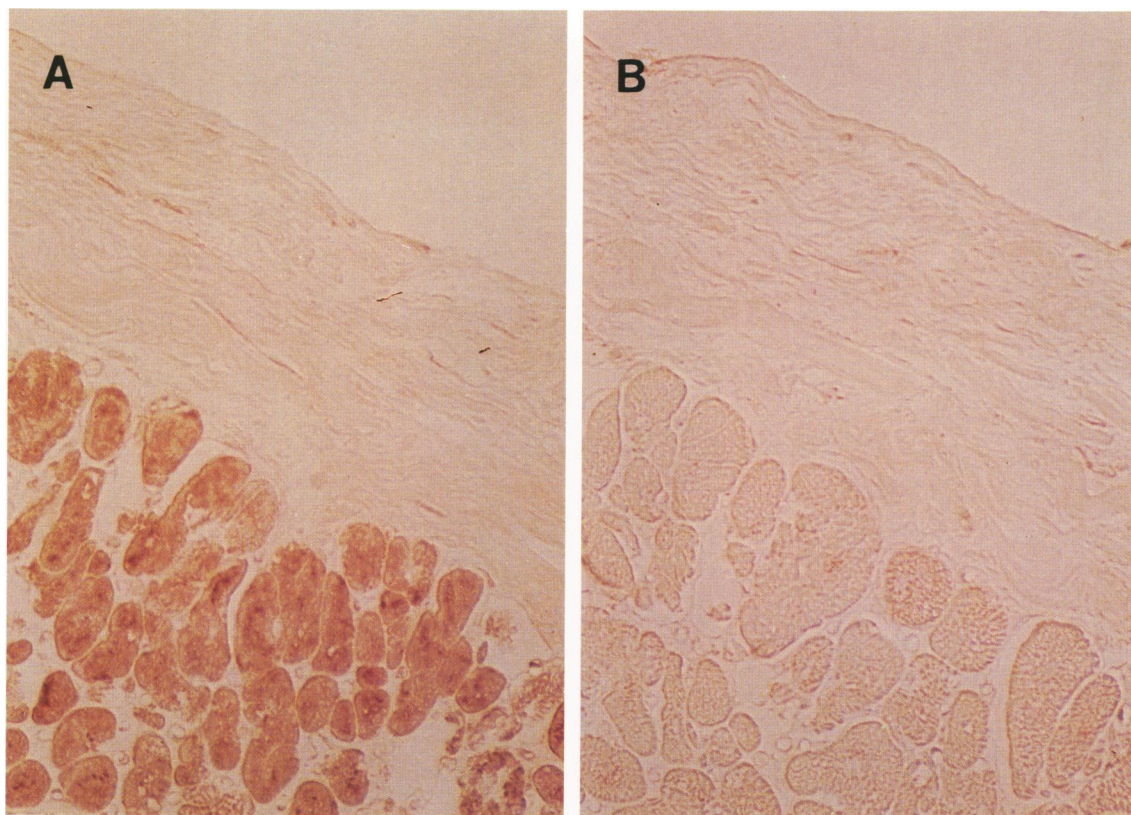
Figure 1. *Continued*

Figure 2. *Normal papillary muscle from a patient of the control group stained for latent TGF-β1 (A) and LTBP (B) using the same method as for the samples in Figure 1. In contrast to the carcinoid plaques shown in Figure 1, no immunoreactivity can be detected in the subendocardial space for both molecules, whereas the staining of the myocardium is the same as in the diseased material. Original magnification is × 250.*

in endothelial cells[14] and in the myocardium[15–18] has recently been reported.

LTBP showed a very different pattern of tissue distribution compared with the TGF-β precursors, both in respect to the staining pattern and in the intensity. The staining for LTBP was always located extracellularly. In some patients, LTBP was clearly detected in the extracellular matrix (Figure 1G), whereas it was negative in all the other cases. Normal control hearts showed no immunostaining in the subendocardial connective tissue (Figure 2B). The myocardium was always negative for LTBP (Figure 1G, 2B).

Discussion

Typical carcinoid heart lesions were present in the right side of the heart in nine patients with carcinoid syndrome undergoing tricuspid and pulmonary valve replacement. We have demonstrated the presence of immunoreactive TGF-β precursors in the carcinoid plaques of all cases and LTBP in a few of them. In contrast, the subendocardial space in corresponding normal heart tissue showed significantly

less or no immunoreactivity for TGF-β at all. These results suggest an induction of TGF-βs during the development of the fibroproliferative lesions in carcinoid heart disease.

The carcinoid plaques contain relatively few cells and consist of deposition of extracellular matrix such as proteoglycans and mucopolysaccharides.[4,5] The fibroblasts present in the plaques are phenotypically immature and express muscle actin in their cytoplasm.[19] TGF-β produced in these plaque fibroblasts might stimulate the production of extracellular matrix in an autocrine fashion. In various other types of fibroproliferative disorders it has been demonstrated recently that TGF-β is involved in the production of extracellular matrix.[6,8,20–22]

Because TGF-βs are synthesized as latent, high molecular weight complexes, effects of TGF-βs in the tissue would require activation of the complexes. The mechanism for the activation *in vivo* is unknown. It is an interesting possibility, which remains to be elucidated, that mechanisms leading to the activation of TGF-β are induced during the development of the fibroproliferative lesion in the carcinoid heart.

The mechanism by which the TGF-β precursors are induced in the fibroblasts of the plaques is still

unknown. It is possible that the vasoactive substances produced by the carcinoid tumor cells trigger the induction. In fact, it has been demonstrated that serotonin and tachykinins, which are frequently produced by carcinoid tumor cells in a high quantity, alone or in combination with other factors, stimulate DNA synthesis in cultured fibroblasts.[23,24]

Among the TGF-β precursors, β3-LAP was most abundantly present within the fibroblasts of the plaques. β1-LAP showed a weaker and less abundant reactivity, but was detectable in most of the sections. β2-LAP stained generally weak and was detectable only in a few cells within the plaques. Further studies are needed to determine whether a different level of expression of the various isoforms of TGF-β reflects functional differences.

The immunostaining of LTBP was partly concordant with the staining for the TGF-β1 precursor, which is known to associate with LTBP in the large latent complex of TGF-β1.[25] This association of the TGF-β1 precursor with LTBP was shown to lead to an efficient secretion of TGF-β1. In contrast, TGF-β1 complexes, which failed to associate with LTBP, remained associated with the producer cell.[26] This is consistent with our findings, which show that LTBP staining was confined to the extracellular compartment and was only detectable in conjunction with increased immunoreactivity of TGF-β precursors. However, most of the carcinoid plaques did not show any detectable LTBP staining. Whether LTBP contains associated TGF-β precursor molecules and whether it associates with the extracellular matrix after being secreted from the producer cell is unknown. The latter possibility is supported by the fact that LTBP has a structural homology to fibrillin,[27] which is an extracellular matrix protein and whose abnormal composition causes a dramatic loss of stability of the connective tissue, as demonstrated in the case of Marfan's syndrome.[28] However, whether LTBP plays a functional role in the extracellular matrix is subject to further investigation.

In conclusion, we have shown that the fibroblasts in carcinoid plaques in the heart express all three TGF-β precursors, suggesting a critical role of TGF-βs in progressive deposition of matrix proteins. It might be feasible to suppress this action *in vivo* by the application of antibodies against TGF-β, as recently demonstrated in another disease.[20] The mechanism by which the TGF-βs are induced, however, needs to be elucidated further.

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